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Report

# Ant Pupae Employ Acoustics to Communicate Social Status in Their Colony's Hierarchy

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## Summary

**The possession of an efficient communication system and an ability to distinguish between young stages are essential attributes that enable eusocial insects to live in complex integrated societies [1–4]. Although ants communicate primarily via chemicals, it is increasingly clear that acoustical signals also convey important information, including status, between adults in many species [5–9]. However, all immature stages were believed to be mute [7]. We confirm that larvae and recently formed pupae of *Myrmica* ants are mute, yet once they are sclerotized, the pupae possess a fully functioning stridulatory organ. The sounds generated by worker pupae were similar to those of workers but were emitted as single pulses rather than in the long sequences characteristic of adults; both induced the same range and intensity of benevolent behaviors when played back to unstressed workers. Both white and sclerotized pupae have a higher social status than larvae within *Myrmica* colonies, but the latter's status fell significantly after they were made mute. Our results suggest that acoustical signals supplant semiochemicals as a means of identification in sclerotized pupae, perhaps because their hardened integuments block the secretion of brood pheromones or because their developing adult secretions initially differ from overall colony odors [5, 10].**

## Results and Discussion

### Pupal Sound Production

The main means of recognition and communication that permit up to a million individuals in an ant society to function as a single "superorganism" is by chemical cues, often modulated by tactile stimuli [1, 2, 4]. Members of the same society typically share a cocktail of hydrocarbons that provides a distinctive "gestalt" odor across the colony, allowing workers to discriminate between kin and strangers [5, 6]. Additional variation between individuals' profiles permits recognition of—and appropriate responses to—nestmates of different sex, caste, and developmental stage [10–14]. For example, when a colony is perturbed, the workers quickly rescue and retrieve the brood, including dummies treated with larval extracts [15, 16]. In the well-studied Myrmicine genus *Myrmica*, brood recognition by pheromones is supplemented by tactile cues, including larval turgidity, hairiness, size, shape, and surface properties [4]; a social hierarchy exists between the different young stages: small larvae are killed and fed to larger larvae in times of food shortage, and a distinct order of rescue occurs—starting with pupae, followed by large larvae and finally by small larvae and eggs—whenever a colony is disturbed [17, 18].

The role of acoustic signaling has recently received increased interest [7–10], but, to our knowledge, there was no evidence that the young stages of any ant could communicate using sound. On the contrary, previous studies indicated that the immature stages of *Myrmica* were mute [7, 19], although older, sclerotized pupae may not have been investigated. Scanning electron microscopy revealed, however, the presence of a fully formed stridulatory organ on the developing imago within sclerotized ant pupae, similar to that on adult workers and queens (Figure 1). The organ consists of a minutely ridged file (Figures 1C and 1D; pars stridens), located on the middorsal edge of the fourth abdominal segment, and of a spike (plectrum) projecting from the rear edge of the postpetiole. However, compared with adults, the scope for the pupa to play one surface rapidly against the other was constrained due to the thin pupal cuticle that encompassed it (Figures 1A and 1B). Emerging stridulatory organs were also recognizable on the soft abdomens of newly formed white pupae but were absent from larvae.

We recorded larvae and white pupae for a total of 40 hr, but no sounds or substrate-borne vibrations were detected. In contrast, sclerotized (nascent worker) pupae readily produced acoustic signals which resembled those of adult workers and, to a lesser extent, queens in their frequency and intensity, but which consisted of single pulses rather than the streams of "song" emanating from both adult castes (Figure 2A). Using a multivariate approach over three sound parameters, the normalized Euclidean distances (mean ± SD) within samples of *M. scabrinodis* pupae, queens, and workers were respectively 0.88 ± 0.32, 0.52 ± 0.30, and 1.00 ± 0.59 (Figure 2A). Principal component analysis (PCA) was also conducted on the three sound parameters recorded from groups of 6 *M. scabrinodis* sclerotized pupae, 1 individual worker, and 1 queen from each of 10 *M. scabrinodis* nests: the first and the second principal components accounted for 79.1% and 20.9% respectively, i.e., explaining all the variance (Figure 2B). Nested analysis of similarity (ANOSIM) of the Euclidean distance matrix showed a clear separation between the signals of sclerotized pupae, workers, and queens (overall: $R = 0.778$, $p < 0.001$; for component distances: sclerotized pupae: $distance_{workers} = 2.52 \pm 1.00$, ANOSIM $R = 0.941$, $p = 0.001$; $distance_{queens} = 3.16 \pm 0.96$, ANOSIM $R = 1$, $p = 0.001$). As expected, the signals emitted by sclerotized (nascent worker) pupae were significantly closer to the stridulations of workers than to those of queens (two-sample t test: $t = 10.198$, $df = 198$, $p < 0.001$).

The adults of many ant species stridulate to nestmates [20, 21], although acoustical communication by their immature stages has not been previously described. Because the active organ formed part of a nascent adult developing inside the sclerotized *M. scabrinodis* pupa, we might expect to find

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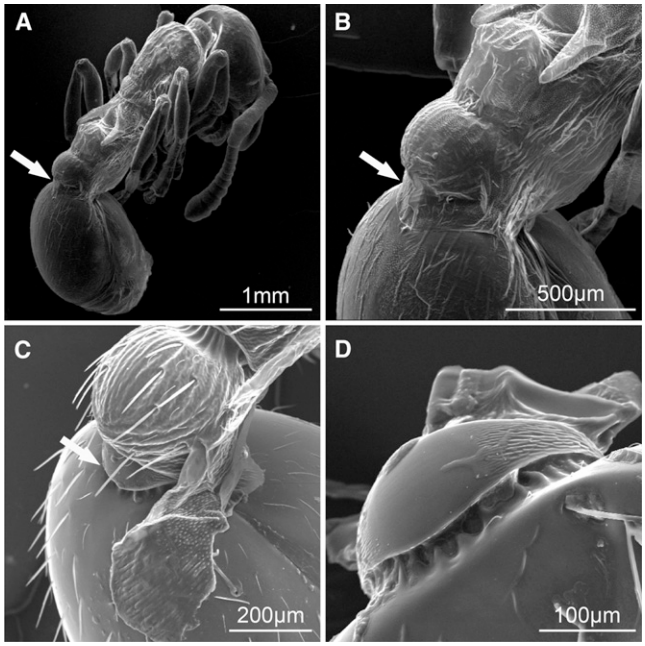


Figure 1. The Stridulatory Organ of Sclerotized Pupae of *Myrmica scabrinodis*

(A and B) Location of the acoustical organ (arrow) beneath the integument of an intact pupa.
(C) Pupa with integument removed.
(D) Pars stridens on pupa with integument removed.

similar acoustical communications, after the first few days of pupal lives, among the four subfamilies of ants which also possess a stridulatory organ, namely the Ponerinae, Nothomyrmecinae, Pseudomyrmecinae, Myrmicinae.

### Worker Ant Responses to Pupal Sounds

The responses of otherwise undisturbed *M. scabrinodis* workers to recordings of the sounds emitted by their sclerotized pupae were compared with playbacks of their own (worker) recordings and of white noise in three randomly assigned containers, simultaneously replicated twenty times.

No antagonistic or alarmed ant behavior occurred during playback experiments, but five benevolent responses were observed, the first two involving attraction and the rest involving reactions: (1) walking—the worker was attracted to the speaker but walked over it without stopping on it; (2) alerting—the worker abruptly changed direction to pass onto the speaker; (3) antennating—the worker antennated the speaker for at least 3 s; (4) guarding—the workers rested in an alert on-guard poise (sensu; [7]) on the speaker for at least 5 s; (5) digging—the worker dug into the soil surrounding the speaker.

Linear mixed-effect models showed that worker reactions to the three sound stimuli were significantly different for all observed behaviors except digging, which, however, was never elicited by white noise (Figure 3). Thus, compared with white noise, both pupal and worker sounds always induced significantly more instances of walking, alerting, antennating, and guarding by *Myrmica* worker ants, with values of p ranging from 0.019 to <0.0001. Yet despite the fact that pupal calls consisted of single pulses, whereas worker stridulations were broadcast in streams, no significant difference was found in worker responses to these two sound stimuli (Figure 3).

The results are consistent with other observations within the genus *Myrmica* that stridulations are caste specific rather than species specific [8], and, unsurprisingly, the structure of the stridulation organ we found in *M. scabrinodis* worker pupae was identical to that of eclosed adult workers (Figures 1 and 2). Similarly, we predict that the stridulatory organ of a gyne pupa will produce sounds similar to an adult queen and will induce similar royal treatment from nurse workers [7]. The constraint of an enveloping integument may explain why the pupal sounds occurred in single pulses rather than the complex repetitions that characterize an adult ant's diagnostic patterns. The fact that both types of adult and pupal stridulations triggered the same intensity and range of benevolent responses suggests that the frequency at which pulses of sounds occur is not important for conveying information. It is worth noting, however, that our test environment was simple and constant, and that in nature adult ants are capable of both producing different sounds [22] and reacting in different ways to the same acoustics [23], depending on the context in which the signal is transmitted or received. Furthermore, our acoustics were tested in isolation, whereas in nature they may be modulated by chemical or tactile cues, and vice versa [1, 2]. Thus, we suspect that tended pupae in natural colonies may possess a wider acoustical repertoire than observed here and that worker responses to them may be more complex.

### Social Status of Normal and Mute *Myrmica* Pupae

As has been reported for other *Myrmica* species [17, 18], we found that *M. scabrinodis* workers rescued living pupae, as

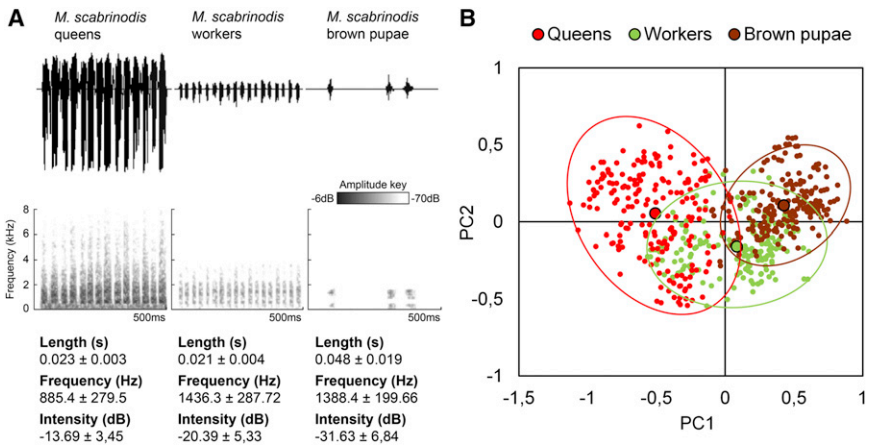


Figure 2. Comparison of the Acoustics of Queen, Worker, and Sclerotized Pupae of *Myrmica scabrinodis*

(A) Oscillogram, spectrogram, and single pulse parameters.
(B) Combined effect of the three sound parameters (pulse length, frequency, and intensity) shown as the first and second component plot of a principal components analysis over all individual pulse measurements.

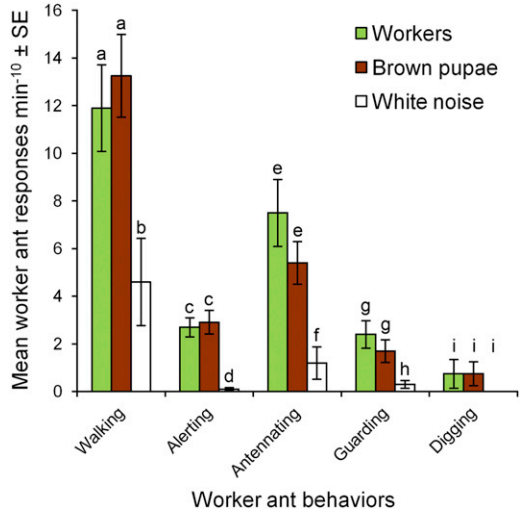


Figure 3. Responses of *Myrmica scabrinodis* Workers to Broadcasts of Worker and Pupal Acoustics and White Noise

Five benevolent but no antagonistic behaviors were observed: the same letter indicates no significant difference within each type of behavior; different letters indicate a significantly different response. Compared with white noise, linear mixed-effect model likelihood ratios are (1) walking $LR_{pupa}$ = 11.082, df = 4, p = 0.001; $LR_{worker}$ = 8.097, df = 4, p = 0.004; (2) alerting $LR_{pupa}$ = 23.232, df = 4, p < 0.0001; $LR_{worker}$ = 20.518, df = 4, p < 0.0001; (3) antennating $LR_{pupa}$ = 8.425, df = 4, p = 0.004; $LR_{worker}$ = 17.154, df = 4, p < 0.0001; and (4) guarding $LR_{pupa}$ = 5.476, df = 4, p = 0.019; $LR_{worker}$ = 11.419, df = 4, p = 0.001. Likelihood ratios comparing pupal and worker acoustics are (5) walking $LR$ = 0.296, df = 4, p = 0.587; (6) alerting $LR$ = 0.145, df = 4, p = 0.704; (7) antennating $LR$ = 2.278, df = 4, p = 0.131; and (8) guarding $LR$ = 1.441, df = 4, p = 0.230.

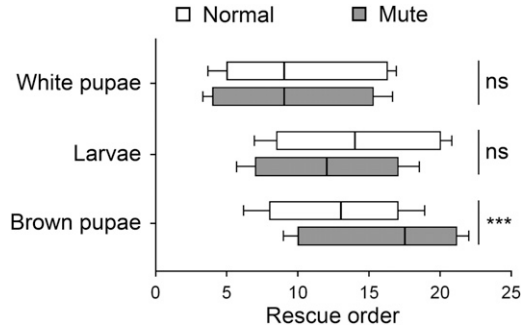


Figure 4. The Hierarchical Status of *Myrmica* Brood Items

Box plots illustrate the order in which worker ants rescued sclerotized (brown) pupae, young white pupae, and larvae after their nest was perturbed by exposure to light: vertical line = median rank of rescue, box = 25th–75th percentiles, whiskers = one standard deviation below and above the mean of the data. White boxes show "normal" live ant brood (overall Kruskal-Wallis $H_n$ = 11.182, df = 2, p = 0.003), and gray boxes show results for "mute" recently dead brood ($H$ = 26.347, df = 2, p < 0.001).

a class (i.e., brown + white), significantly more quickly than their larvae (Wilcoxon Mann-Whitney, $Z$ = 6.822, p = 0.009) after their nest was disturbed (Figure 4). However, within these assays, using normal (i.e., nonmuted) brood items, the white pupae were rescued ahead of both sclerotized pupae ($Z$ = 2.118, p = 0.026) and larvae ($Z$ = −3.177, p < 0.001), with no significant difference being found between sclerotized pupae and larvae ($Z$ = −1.399, p = 0.168), although the latter were, on average, rescued last (Figure 4).

The pattern of rescue changed with recently killed brood: i.e., brood still coated with its full cocktail of recognition pheromones [24, 25] but which was mute and immobilized (Figure 4). The mute sclerotized pupae were the last to be rescued, significantly behind white pupae ($Z$ = 3.326, p < 0.001) and larvae ($Z$ = 2.306, p = 0.021). White pupae were on average rescued first, but not significantly ahead of larvae ($Z$ = 1.5875, p = 0.107). Wilcoxon signed rank tests were also used to directly compare the shift in order in each brood type between the normal and mute trials: sclerotized pupae shifted to being rescued significantly after the other brood in the mute trials ($Z$ = −24.500, df = 10, p = 0.0098), but there was no significant shift in the order of recovery of white pupae or larvae between the two experiments ($Z$ = −4.500, df = 10, p = 0.6719 and $Z$ = −15.500, df = 10, p = 0.1309, respectively).

It was impractical to record the acoustics of *Myrmica* pupae during the rescue experiment, but the shift in rank for the brown pupae that could and could not stridulate indicates that this is linked to the stridulations. Of course, dead brood cannot move, either; e.g., larvae cannot beg, but the lack of any significant difference in the relative order of rescue of white pupae and larvae during the mute assays compared with the living trials supports previous conclusions [24, 25] that the chemical and tactile signals involved in brood recognition are not compromised by this treatment.

The preference afforded to living white pupae after colony perturbation was unexpected. We predicted that the calls of sclerotized pupae would attract preferential worker attention, perhaps explaining why pupae as a group were selected ahead of larvae or eggs in previous ant rescue experiments [17, 18]. A possible explanation is that, rather than elevating the social level of sclerotized pupae through the possession of an additional cue, their acoustics may replace brood-recognition pheromones, perhaps because the hardened integument blocks the secretions from their own glands or reduces their ability to absorb colony odors. An alternative explanation is that hydrocarbons secreted by the developing imago within a sclerotized pupa not only replace or overscore the pheromones of brood with soft cuticles, but—like the secretions of the callow adults that they will shortly become—differ somewhat from the overall gestalt odor of their colony, making them less recognizable as nestmates using chemical cues alone.

## General Conclusions

Our results support a growing body of work—facilitated by the increased sophistication of affordable sound equipment—that suggests that acoustical communication plays a greater and more varied role in influencing ant social behavior than was previously thought (e.g., see [7, 8, 20–22, 26]).

The recognition, not only of brood in ant societies but also of different types of brood, including nestmate and nonnestmate brood, has received much recent attention. Although it seems clear that chemical, tactile, behavioral, and now acoustic cues can be important in brood recognition [4, 10, 25], the precise role of each cue is still poorly understood. For instance, on current evidence we have suggested that acoustical signals are caste specific but not species (let alone kin) specific. On the other hand, the cuticular hydrocarbon signatures described on brood are often impoverished and dominated by saturated alkanes that are not thought to convey information [27, 28]. If this were the case, brood would be chemically transparent [10, 29] and distinctive to workers only if other

undescribed brood pheromones exist [30]. Recent studies, however, have shown that nonnestmate brood is often adopted into an ant society as a quick and efficient way of increasing the workforce, whereas behavioral experiments show that any kin brood is always chosen first, indicating a clear ability among workers to recognize immature nestmates within their species [10, 31].

Notwithstanding the predominant use of semiochemicals in ant communications, many species generate acoustical signals through a stridulatory organ or by drumming their gaster. Once considered a weak form of communication, restricted to spreading alarm or modulating responses to other signals [1, 32–34], it is increasingly clear that acoustics is used to convey a greater variety of information between nestmates as well as to signal an individual's social status [7, 8]. We suspect that acoustics may be a more flexible means of signaling and conveying information between both adult and immature ants than is generally recognized [22].

## Experimental Procedures

### Field Collection and Culture

*Myrmica scabrinodis* nests (n = 10) were collected in July 2011 at Wallingford (UK), set as standardized laboratory ant colonies with >100 workers in 12.5 cm × 8 cm × 2 cm Perspex containers, and maintained on a diet of sugar and *Drosophila* larvae [35]. All colonies contained a minimum of ten larvae, ten white pupae, and ten sclerotized pupae.

### Scanning Electron Microscopy

We used dissection and scanning electron microscopy to investigate the presence of stridulatory organs on ant brood. Two *M. scabrinodis* larvae and two white and two sclerotized pupae from two ant colonies were kept in 70% ethanol, and one item per category was dissected between the postpetiole and the abdomen to expose the pars stridens and the plectrum. The whole individuals and the two ant parts were mounted on the same steel stub and coated with gold, and the samples were scanned using a Cambridge Stereoscan S360 scanning electron microscope. *M. scabrinodis* white pupae and larvae were dried in hexamethyldisilazane to avoid cell structure disruption before coating. The SEM operated at 20–25 kV.

### Sound Recordings

We recorded sounds of clusters of six *M. scabrinodis* larvae and six white and six sclerotized pupae from ten *M. scabrinodis* nests. Separate recordings were made of individual queens and workers taken from the same test colonies. The recording equipment consisted of a 12.5 cm × 8 cm × 2 cm recording chamber with a moving-coil miniature microphone attached through the center. A second microphone of the same type was used to record ambient noise but in antiphase. An amplifier was attached to each microphone and calibrated to maximize the noise cancellation of ambient noise from the two microphones, leaving the signal from the recording chamber. The resulting signal was processed through two-stage low-noise amplification before being digitally recorded on a laptop computer, using Audacity 1.3 Beta (http://audacity.sourceforge.net/). To further reduce ambient noise and interference, the equipment was powered by a 12 V gel cell battery, and the recording chamber and microphones were placed inside an anechoic chamber. Sounds were recorded for 20 min periods starting 10 min after items were introduced into the recording chamber.

Recordings were sampled at 44.10 kHz and 32-bit resolution. Frequency information was obtained through fast Fourier transformation (FFT; width 1,024 points). Spectrograms were obtained at Hanning window function with 512 bands resolution. We selected 20 good quality pulses from each track and measured dominant frequency (Hz), pulse length, and sound amplitude (dB) using Audacity 1.3 Beta. Based on the three sound parameters, single pulses were ordinated by principal components analysis (PCA). To test whether sound differed between groups, we calculated the pairwise normalized Euclidean distance over all three parameters and used a nested ("colony" within "group") ANOSIM implemented in Primer v6 (Primer-E Ltd.). The sound parameters were log(x+1) transformed. We calculated the average pairwise distances and used a two-sample t test to compare differences between group distances.

### Worker Ant Responses to Sound Recordings

Behavioral assays were carried out in three 7 cm × 7 cm × 5 cm Perspex arenas with the speaker attached at the bottom of the box and sealed on the outside with Blu-Tack. The speaker was covered with a thin layer of slightly wet soil. Ten workers from the same *M. scabrinodis* colony were placed in each arena and allowed to settle for 10 min before being played one of the three test sounds (*M. scabrinodis* worker, sclerotized pupae sound, and white noise). The sounds were produced by MP3 players playing loops of the original recordings, with each volume adjusted to the natural level by attaching the speaker to the microphone of the recording equipment and by calibrating to the same levels reached during recording. Each trial lasted 30 min: counts were made of all instances of antagonistic or attractive behaviors, during periods of one minute for each box, and in sequence between the three treatments, i.e., Σ10 min for each sound per trial. Each playback experiment was repeated 20 times, using fresh ants from ten different *M. scabrinodis* colonies (i.e., twice for each colony). The source of sound for each arena was randomly assigned before each trial was replicated to control for possible positional effects. Between each trial, new soil was introduced and all the equipment, including speakers and arenas, was cleaned with absolute alcohol and rinsed with distilled water. The effect of sound stimulus on the five worker ant behaviors was analyzed in a linear mixed-effects model with "colonies" as a random factor using the software R-2.15.0 [36].

### Experiment to Measure the Order in which Workers Rescued Different Brood Items

The arena used for the brood-rescue experiment consisted of two adjacent chambers of 7 × 2 cm communicating at one end. We placed eight *Myrmica* larvae, eight white pupae, eight sclerotized pupae, and ten workers on a 0.4 $cm^3$ moist sponge (to maintain humidity) at the end of one chamber, which was then covered with a transparent glass. The other chamber was covered with a dark glass. After 10 min of resting in the dark, we shone a 60 W light placed 10 cm away onto the chamber containing the worker ants and brood, to create a high level of stress which induced workers to rescue the exposed brood and carry it into the dark chamber. The order in which each item of brood was rescued was recorded. The experiment was then repeated after placing all brood items from a colony in a freezer (−20°C) for 20 min, thus killing the brood to make them mute (and immobile). Brood items were then left at room temperature for 5 min to return to normal temperature. Immediately after this period, the same procedures as before were used to make rescue experiments. Previous studies [24, 25] have established that in assays conducted only a short time after immature ants are killed, the chemicals responsible for brood recognition remain present in approximately the same quantities as in the live brood.

Statistical analyses were performed using the package "coin" provided with the software R-2.15.0 [36, 37]. Kruskal-Wallis tests were used to compare the rescue orders of different brood categories between nonmute and mute treatments. Subsequent pairwise comparisons of median rescue order between brood categories within the same treatment were made using Wilcoxon Mann-Whitney tests; p values were calculated against a null distribution generated from data using a Monte Carlo resampling. Direct comparisons of the same brood categories between normal and mute treatments were made using paired Wilcoxon signed rank tests.

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